Antioxidant Activity and Cytotoxicity of *Taraxacum hispanicum* Aqueous and Ethanolic Extracts on HepG2 Cells

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ABSTRACT

Introduction: Plants belonging to the genus *Taraxacum* have been used in traditional medicine. Nowadays, extracts of these plants have been reported for the treatment of diseases, including liver disorders. Increasing interest and research on these plants also revealed its potential for treating cancer. This study aims to evaluate the antioxidant activity and cytotoxic properties of crude extracts from aerial parts of *Taraxacum hispanicum* H.Lindb, against human hepatocarcinoma (HepG2). Material and methods: Evaluation of the antioxidant properties was performed using DPPH *in vitro* test, superoxide scavenging assay and Fe^{2+} chelating activity. MTT assay was used to determine metabolic activity, for 24 and 48 hours. Results: For antioxidant capacity of the ethanolic extract (overall the one with the best results), IC_{50} values were 62.4 ± 6.7 µg/ml (DPPH radical scavenging activity) 53.9 ± 10.3 µg/ml (Fe^{2+} chelating activity) and 2.0 ± 0.3 µg/ml (superoxide scavenging assay). The aqueous and ethanolic extracts had different effects on HepG2 cell viability. Aqueous extract induced cell cytotoxicity in a time and dose-dependent manner, leaving only 52.6% viable cells at a concentration of 200 µg/ml, after 48 h. An increase in the cell viability was seen in the ethanolic extract, from 24 h to 48 h at higher concentrations. Conclusions: Ethanolic extract of *T. hispanicum* was the most promising, presenting anti-oxidative capacity and only the aqueous extract of the plant presented more relevant cytotoxicity over HepG2 cell line. These activities may be related with the extract phenolic content. However, further studies are needed to elucidate the main mechanisms responsible for these potential effects.

Keywords: Antioxidant, cytotoxicity, HepG2 cells, *Taraxacum hispanicum*.

INTRODUCTION

Plants have been used for centuries by people of all cultures for treating various ailments. It is estimated that 80% of the world's population living in developing countries rely on plants as a primary source of healthcare and traditional medical practice[1]. The use of medicinal plants over the years has attracted the attention of scientists' worldwide[2]. However, although some plants have promising potential and are widely used, many of them remain untested[3].

Reactive oxygen species (ROS) are highly reactive molecules derived from the metabolism of oxygen. ROS induce lipid peroxidation, damage biomolecules, and affect cellular viability[4]. The uncontrolled generation of ROS often correlates directly with molecular markers of many diseases, including cancer[5]. The formation of cancer cells in the human body can be directly induced by free radicals[6].

Antioxidants are substances capable of neutralize the excess of free radicals and protect cells against their toxic effects[7]. Natural antioxidants, found in plants, can protect cells from oxidative stress by preventing the formation or detoxifying free radicals, resulting in the prevention of a variety of pathophysiological processes[8]. Therefore, the knowledge and identification of natural products that could limit ROS-mediated injuries and that could act as chemotherapeutic and chemopreventive agents has gained popularity.

Plants of the genus *Taraxacum* play an important role in Chinese, Arabian and Native American traditional medicine, and their leaves and roots are frequently used to treat lung, breast and uterine tumors as well as hepatitis and some digestive diseases[9,10].

Taraxacum officinale (L.) Weber ex F.H. Wigg (family Asteraceae), also known as dandelion, is the most studied species, and seems to be the most consumed both as food and in traditional medicine[11]. The plant contains flavonoids, triterpenes, coumarins, and phytosterols[12]. Some studies have revealed that extracts of *T. officinale* possess antioxidant, hepatoprotective, anti-inflammatory, and antitumoral activities[4,12,13,14]. Although plants of the genus *Taraxacum*, including *T. officinale*, are often used

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in traditional medicine for the treatment of cancer, scientific evidence to support this effect is lacking. Hata et al.,[15] found that upon screening a variety of compounds from wild plants, *T. officinale* was an effective inducer of differentiation in mouse melanoma cells. Koo et al.,[16] showed that antitumor action has been demonstrated for aqueous *T. officinale* extracts, through TNF-α and IL-1α secretion in HepG2 cells. In another study, Sigstedt et al.,[17] also demonstrated that the crude extract of dandelion leaf decreased the growth of MCF-7/AZ breast cancer cells in an ERK-dependent manner. Chatterjee et al.,[13] showed that dandelion root extract specifically and effectively induces apoptosis in human melanoma cells without inducing toxicity in noncancerous cells. More recently, Yoon et al.,[18] confirmed that combination treatment of Huh7 cells with TNF-related apoptosis induced ligand (TRAIL) and *T. officinale* led to TRAIL-induced apoptosis.

Studies on the anti-oxidative capacity of *T. officinale* showed that both root and leaf extracts diminish lipid peroxidation and reduce cytochrome c[19]. The content of phenolic compounds in dandelion extracts was responsible for the inhibition of reactive oxygen species and nitric oxide induced damage[20].

The active phytochemicals of *T. officinale* (the most studied species of the genus) are found in both roots and leaves. Chemical constituents present in the dandelion leaves are bitter sesquiterpene lactones, several polyphenols and coumarins[21]. Other compounds include β-amyrin, taraxasterol and taraxerol as well as free sterols such as sitosterin, stigmasterin, and phytosterin[9]. Polyphenols are the subject of increasing scientific interest because of their beneficial effects on human health[22]. Some polyphenols like chicoric acid, caffeic acid, luteolin, luteolin 7-glucoside, rutin, and apigenin have been identified in the genus *Taraxacum*[11,21].

Due to genus morphology, determination of the correct *Taraxacum* species is very difficult for a non-expert. Furthermore, because *T. officinale* is not present in the South of Europe, other common *Taraxacum* species are being used as traditional medicine in these areas[11]. To our knowledge, few studies have been reported addressing the cytotoxic effects of *T. hispanicum* extracts, on different cell lines. This study aims to evaluate whether dried aqueous and ethanolic extracts of aerial parts of the plant have an antioxidative and cytotoxic effect on HepG2 cells.

MATERIAL AND METHODS
Chemicals
Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic solution, trypsin solution, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT), reduced form of nicotinamide adenine dinucleotide (NADH), N-phenylmethazonium methosulfate (PMS), quercetin, ferrozine, and all other not specified reagents were from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biochrom KG (Berlin, Germany). L(+)-

Ascorbic acid and pyruvate were purchased from Panreac (Barcelona, Spain). Ethylenediaminetetraacetic acid (EDTA) and dimethyl sulfoxide (DMSO) were purchased from VWR (Portugal). All other chemicals were of analytical reagent-grade.

Plant material and preparation of the extracts
The aerial parts of *T. hispanicum* used in this study were collected from Vila Nova de Gaia (Portugal) during May 2016, identified and authenticated by a specialist at Sciences Faculty, University of Porto, Portugal. A voucher specimen was kept in the herbarium of the institution (PO-V62372). The plant parts were washed under running tap water to remove dirt and soil and finally rinsed with distilled water.

The aqueous extract of *T. hispanicum* for *in vitro* screening was prepared as follows: 75 g of the dried leaves were soaked in water for 24 h at room temperature and in the darkness. The mixture was filtered through a paper filter (Whatman, No. 1), to remove particulate matter, lyophilized and the resulting powder was stored at 4˚C for further use. Freeze-drying conditions were of 0.008 mBar, for 3 days, with a condenser surface temperature of -77ºC.

For the ethanolic extract of *T. hispanicum* 75 g of the dried leaves were macerated with an ethanol-water solution (80:20, V/V), at room temperature and in the darkness for 4-5 days. The mixture was then filtered through a paper filter (Whatman, No. 1), and concentrated to obtain the crude extract. Stock solutions of test extracts were made in dimethylsulfoxide (DMSO) and aliquots were kept at -20ºC for further use.

Antioxidant Activity
DPPH radical scavenging activity
The measurement of the *T. hispanicum* extract scavenger activity against the DPPH radical was performed in accordance with Lima et al.,[23] with modifications[20]. DPPH˙ solution (90 μM) was added to a medium containing different concentrations of the aqueous and ethanolic extract (1, 5, 10, 50, 100, and 200 μg/mL) or vehicle (EtOH) and incubated in the dark, at room temperature, for 30 min. The reduction of DPPH absorption was measured at 515 nm using a plate reader spectrophotometer. Quercetin was used as a positive control. Radical scavenging ability was calculated using the following formula: radical scavenging ability (%) = 100 x [(AC-AS)/AC], where AC is the absorbance of the control, and AS is the absorbance of the sample (extract or standard). The concentration of the plant extract/control required to scavenge 50% of the total DPPH radicals (IC_{50}) available was calculated.

Superoxide radical scavenging activity
Superoxide radical scavenging activity was determined using the PMS-NADH nonenzymatic assay as previously described[24]. Briefly, the reaction mixture in the sample wells consisted of NADH (166 μM), NBT (43 μM), *T. hispanicum* extracts and PMS (2.7 μM) in 19 mM phosphate buffer, pH 7.4. The assay was performed at room temperature during 2 min and started with the addition of PMS. The absorbance was determined over time at 560 nm. Ascorbic acid was used as positive

control. Superoxide radical scavenging ability was calculated using the following formula: superoxide radical scavenging ability (%) = 100 x [(AC-AS)/AC], where AC is the absorbance of the control, and AS is the absorbance of the sample (extract or standard). The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against extracts concentrations.

Fe^{2+} chelating activity

Iron chelating activity of the *T. hispanicum* extracts was evaluated according to Russo et al.,[25] with some modifications. Briefly, 50 μl of the extracts at different concentrations were added to a solution of 0.12 mM of ferrous sulphate ($FeSO_4$) (50 μl) and 50 μl of ferrozine (0.6 mM). The mixture was then shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured at 562 nm in a spectrophotometer. EDTA was used as a positive control. The ability of *T. hispanicum* extracts to chelate ferrous ion was calculated relative to the control using the formula: chelating activity (%) = 100 x [(AC-AS)/AC], where AC is the absorbance of the control, and AS is the absorbance of the sample (extract or standard). The IC_{50} value (μg/ml), which is the concentration of the extract/standard that chelates 50% of the ferrous ion, was calculated.

Cell culture

The human hepatocellular carcinoma (HepG2) cell line was obtained from the American Type Culture Collection (ATCC), and maintained in 25 cm^2 polystyrene flasks in DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic solution, 1 mM sodium pyruvate, 10 mM HEPES and 1.5 g/l sodium bicarbonate. Cells were grown at 37°C in a humidified incubator with 5% CO_2. When the cells reached at >80% confluency, subculture was conducted at a cell density of $2.0x10^6$ cells/well. The medium was refreshed three to four times a week.

Assay for cell viability

Cell viability of HepG2 cells was estimated using the MTT colorimetric assay[26]. Briefly, cells were seeded (2.0×10^5cells/well) into 24-multiwell culture plates and cultured at 37°C in a humidified atmosphere of 5% CO_2. The plate was incubated for a period of time to assure attachment and 40% to 60% confluency. Cells were then treated with increasing concentrations of each *T. hispanicum* extract (ranging from 1 to 200 μg/ml), in fresh complete medium for 24 and 48 h.

After the exposure time, MTT solution (final concentration 0.5 mg/ml) was added to each well and incubated for more 1 h. Then, MTT-containing medium was removed gently and replaced with a 50:50 (V/V) DMSO:ethanol solution to mix the formazan crystals until dissolved. Presence of viable cells was visualized by the development of purple color due to formation of formazan crystals. The absorbance was measured at 570 nm using a 96-well microplate reader. The cytotoxicity was expressed as the percentage of control, which had no sample. For each extract tested, the IC_{50} (concentration of drug needed to inhibit cell growth by 50%) was generated from the dose-response curves for HepG2 cell line, using

the GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, USA).

Statistical Analysis

Data are presented as mean ± SD of at least three independent experiments. In the *in vitro* cell assays One-way ANOVA was employed with Dunnett's multiple comparison test, when comparing each concentration against a control and unpaired t test when comparing two similar concentrations, using GraphPad Prism 5.0. Differences were considered to be significant at a level of $p < 0.05$. In the *in vitro* antioxidant assays unpaired Students t test was used. The IC_{50} was calculated from the dose-response curve obtained by plotting the percentage of inhibition versus the concentrations (in logarithm), using GraphPad Prism 5.0.

RESULTS

In this study, antioxidant activities of the aqueous and ethanolic extracts of *T. hispanicum* were evaluated by applying three common methods, DPPH, iron chelating assay and superoxide radical scavenging activity. All extracts presented different antioxidant activities (Table 1).

In the measurement of *T. hispanicum* extract scavenger activity against the DPPH radical, ethanolic extract showed significant better results ($IC_{50} = 62.4 ± 6.7$ μg/ml) when compared to aqueous extract (p < 0.001) ($IC_{50} = 124.5 ± 2.3$ μg/ml). According to the results, both plant extracts are not as good as the standard quercetin ($IC_{50} = 1.85 ± 0.2$ μg/ml).

Regarding iron chelating activity, best IC_{50} value was shown by aqueous extract ($IC_{50} = 49.7 ± 11.1$ μg/ml) when compared to ethanolic extract ($IC_{50} = 53.9 ± 10.3$ μg/ml). Again, and according to the results, both plant extracts are not as good as the standard EDTA ($1.2 ± 0.0$ μg/ml).

Analyzing the results of the superoxide radical scavenging activity, best IC_{50} value was shown by ethanolic extract ($2.0 ± 0.3$ μg/ml), being significantly lower (p < 0.0001), when compared with aqueous extract ($48.8 ± 3.2$ μg/ml). In both cases, the IC_{50} values were lower than the positive control, ascorbic acid ($IC_{50} = 77.5 ± 10.9$ μg/ml), a well-known antioxidant.

To evaluate the cytotoxic activity of aqueous and ethanolic extracts of *T. hispanicum*, against HepG2 cell line, cells were incubated with different concentrations (ranging from 1 to 200 μg/ml) of extract. After 24 and 48 h of incubation, cell viability was determined by MTT assay. For the aqueous extract, HepG2 cells experienced a significant decrease in viability at the highest concentrations of the extracts, after 24 and 48 h. The extract induced cell cytotoxicity in a time and dose-dependent manner, as presented in Figure 1. Based on metabolic activity of HepG2 cells, treatment with 200 μg/ml of the aqueous extract resulted in 7.5% and 47.4% reduction in cell viability against control within 24 h and 48 h, respectively (Fig. 1).

The aqueous and ethanolic extracts had different effects on HepG2 cell viability, with aqueous extract being more toxic after 48 h of exposure, leaving only 52.6% viable at

Table 1: Antioxidant activities of studied plant extracts from *T. hispanicum*.

Plant Extract	DPPH free radical scavenging	Fe^{2+} chelating ability	Superoxide radical scavenging activity
	IC$_{50}$ value (µg/ml)		
Aqueous extract	124.5 ± 2.3	49.7 ± 11.1	48.8 ± 3.2
Ethanolic extract	62.4 ± 6.7[#]	53.9 ± 10.3	2.0 ± 0.3[¥]

n = 3; results are showed as mean ± standard deviation
[#]significantly different ($p < 0.001$)
[¥]significantly different ($p < 0.0001$)

a concentration of 200 µg/ml (Fig. 1).
On the other hand, in the ethanolic extract the percentage of living HepG2 cells significantly increased from 79.1% (at 24 h) to 119.0% (at 48 h) at 200.0 µg/ml, as presented in Figure 2. An increase in the cell viability was seen only in the ethanolic extract, from 24 h to 48 h at higher concentrations (25 to 200 µg/ml).

DISCUSSION
In this study, we evaluate antioxidant activities of aqueous and ethanolic extracts of *T. hispanicum* by applying different methods, based on single electron transfer mechanisms (DPPH), metal ion chelation and free radical scavenging.
Antioxidant properties found in plant extracts have been attributed to polyphenols[27]. Polyphenols act as antioxidants through several mechanisms, like radical scavenger, metal-chelating agents, hydrogen donor, or electron donor[28].
DPPH assay is a decolorization method capable of measuring the relative antioxidant abilities of natural compounds and extracts to scavenge free radicals. It is a commonly used antioxidant method due to excellent reproducibility, stability, commercial availability and by the fact that it is an easy measurement method[29]. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen-donating ability[30].
Superoxide radical is formed in almost all aerobic cells as a result of one oxygen electron donation, being relevant in the oxygen toxicity mechanism[31]. By itself, superoxide is a week radical but it may cause severe damage to the cell by generating hydroxyl radical and singlet oxygen[32]. The scavenging activity of *T. hispanicum* extracts was established by the NBT reduction method, where the non-enzymatic PMS/NADH system generates superoxide radicals, which reduce the yellow NBT^{2+} in order to produce the purple NBT formazan. Antioxidants have the ability to inhibit purple NBT formation trough competition with NBT for superoxide radical[31].
Iron can stimulate lipid peroxidation by the Fenton reaction and by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can perpetuate chain of reaction. Metal chelating capacity is therefore significant since it reduces the concentration of the transition metal that catalyzes lipid peroxidation. Ferrozine produces a violet complex with Fe^{2+}. In the presence of a chelating agent, complex formation is interrupted and as a result the violet color of the complex is decreased[33].
In this study, ethanolic extract showed better results in DPPH and superoxide radical scavenging activity, as

presented by IC$_{50}$ values (Table 1). In their study, Mingarro et al.,[11] showed that all extracts studied (*T. obovatum*, *T. marginellum*, *T. hispanicum*, *T. lambinonii*, and *T. lacistrum*) had a moderate capacity to scavenge the DPPH radical.
The best DPPH radical scavenging observed in ethanolic extract of *T. hispanicum* is thought to be due to their hydrogen donating ability[34]. Hu & Kitts[20] showed that DPPH radical scavenging activity in the *T. officinale* flower extract was attributed to a reducing activity derived from the phenolic content of the plant. Also, in their study Hu & Kitts[20] found that the superoxide radical inhibition of dandelion flower extract was associated with a direct affinity to scavenge superoxide radical, and this observed inhibition could be attributed in part to the phenolic content. Polyphenolic compounds may react with the superoxide radical, via a one-electron transfer, or by a hydrogen abstraction mechanism[35].
Several plant extracts exert their antioxidant activity by chelating metals[36]. The metal chelating ability of flavonoids is dependent on their unique phenolic structure and position of hydroxyl groups[37]. In this study, both extracts did not show relevant Fe^{2+} chelating activity. The majority of compounds present in the extracts are, therefore, unlikely to have the require structure and/or to exist in a sufficient amount to exert this ability, explaining the results observed.
Phenolic compounds, such as luteolin, luteolin-7-glucoside, caffeic acid, chicoric acid, rutin and apigenin are found in extracts of *T. hispanicum*[11,14]. Compared to roots, dandelion leaves are characterized by higher polyphenol contents[21]. The most abundant phenolic compounds in leaves and flowers are hydroxycinnamic acid derivatives[21,38]. It has been shown that chicoric acid, a derivative of caffeic acid, has the most powerful antioxidant activity among reference compounds such as caffeic acid or rosmarinic acid[39]. Various flavonoid glycosides such as 7-*O*-glycosides of apigenin, rutin, and luteolin were identified in *T. hispanicum* aerial parts[11]. Therefore, the antioxidant activities of plant extracts demonstrated in this study, especially by the ethanolic extract, could be explained by the presence of these compounds, being therefore, related to solvent polarity and solubility of compounds in tested solvents[40].
Cellular viability is affected by oxidative stress, since the production of reactive species causes damage to the inner and outer mitochondrial membranes and opens the mitochondrial permeability transition pores, thereby inducing apoptosis[41]. In this study, the MTT assay was used to estimate the cell viability. The assay is based on
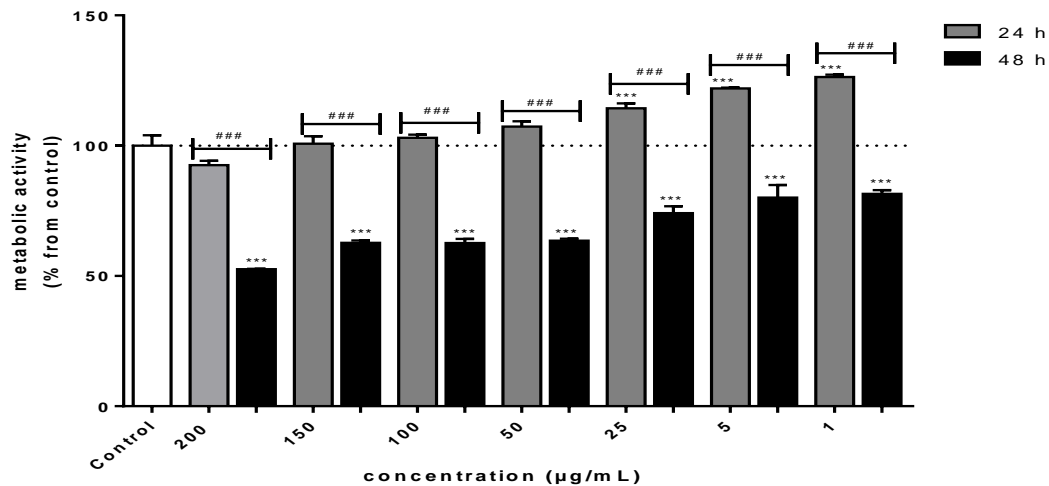
Figure 1: Cytotoxicity of different concentrations of aqueous extract, ranging from 1.0 to 200.0 μg/ml, from *T. hispanicum* in HepG2. Cell viability was determined by MTT assay. Data are presented as mean ± SD of at least three independent experiments, and significant differences as compared with the control group were established at ***p < 0.001; ###p < 0.001.
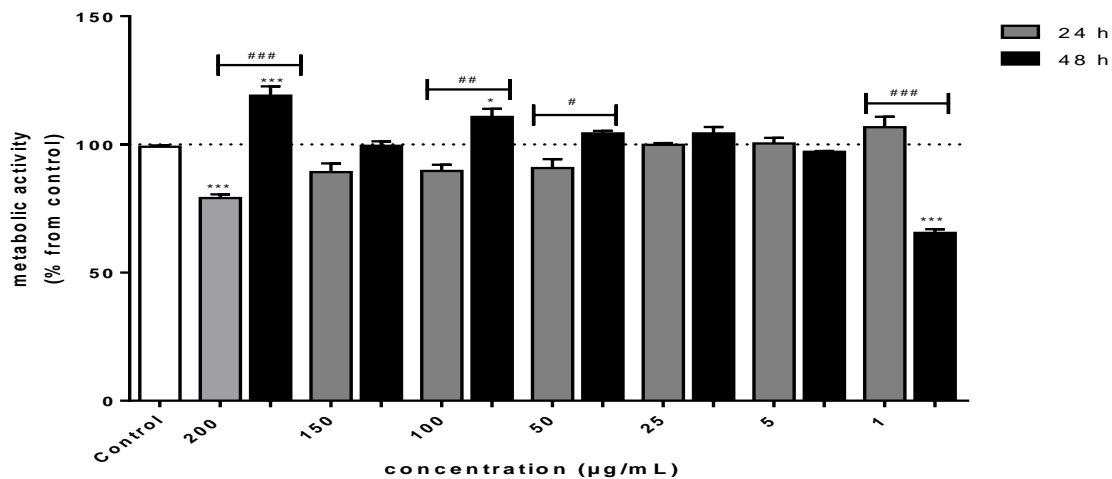

Figure 2: Cytotoxicity of different concentrations of ethanolic extract, ranging from 1 to 200 μg/ml, from *T. hispanicum* in HepG2. Cell viability was determined by MTT assay. Data are presented as mean ± SD of at least three independent experiments, and significant differences as compared with the control group were established at *p < 0.05, #p < 0.05, ##p < 0.01, ***p < 0.001, ###p < 0.001.

the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble tetrazolium salt MTT to purple formazan crystals (insoluble in water) only by viable cells[42]. HepG2 cells, which are easy to handle, retain many of the morphological characteristics of liver parenchymal cells[43], and contain several enzymes responsible for the activation of various xenobiotics[44,45].

Aqueous extract of *T. hispanicum* significantly induced cell death of human hepatocellular carcinoma cells. Aqueous extracts of *T. officinale* have been reported to decrease the growth and invasive ability of breast cancer cells[17]. Similar results were presented by Koo et al.,[16] where it was found that aqueous *T. officinale* extract cause a time-dependent and partially dose-dependent reduction in cell viability. In their study, at 0.2 mg/ml and 48 h, cell viability of the *T. officinale* treated group was

74% (P < 0.05) of the control. The authors also found that *T. officinale* induced the secretion of TNF-a and IL-1a and then induced apoptosis of HepG2 cells. The inhibitory effect may result from phenolic compounds as dandelion leaves are characterized by higher polyphenolic acids and flavonoid contents[46]. This is further supported by studies that describe the effect of polyphenols on cancer cell proliferation[47]. Hu & Kitts[14] demonstrated that the antioxidant and cytotoxic properties of dandelion flower extracts can partly be attributed to the presence of luteolin and luteolin 7-O-glucoside. The aqueous extract of *T. officinale* also has high contents of chicoric acid that may be responsible for the cytotoxic activity[48]. For example, the biological properties of chicoric acid have been reported to include anticancer and antiviral[49,50].

Some important components of the *T. officinale* extract include sesquiterpene lactones and phenylpropanoids,

which are believed to have anti-cancer properties leading to diverse observed effects of dandelion extracts[51]. However, other components of the plants of *Taraxacum* genus have not been fully characterized and therefore their activities remain unknown[46,52].

The higher polyphenol content of the leaves compared to root extracts of *T. officinale*, also proved to be effective in various *in vitro* systems for the determination of antioxidant properties and radical scavenging capacity[19]. These antioxidant properties may be related to the increase in cell viability with time (from 24 to 48 h) observed with ethanolic extract in almost tested concentrations (Fig. 2), suggesting a potential hepatoprotective effect of this extract in HepG2 cells. In their study, Colle et al.,[53] showed that ethanolic *T. officinale* leaves and root extract significantly attenuated marker enzymes of liver toxicity (aspartate transaminase and alanine transaminase), lipid peroxidation and oxidative stress induced by acetaminophen in mice. These protective effects of *T. officinale* have been suggested to the presence of phenolic compounds in the extract.

CONCLUSION

In this study, the antioxidant properties and cytotoxic properties of aqueous and ethanolic extracts from *T. hispanicum* aerial parts, against human hepatocarcinoma (HepG2) were evaluated. Ethanolic extract, in general, revealed better antioxidant and cytoprotective properties, in comparison with the aqueous extract. Aqueous extract proved to be more toxic, especially after 48 h incubated with HepG2 cells. The results observed seem to be related with the extracts phenolic content. However, further studies are needed to elucidate the main mechanisms responsible for these potential effects.

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